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Cloning of *Plasmodium yoelii* genes expressing three different sporozoite-specific antigens

A. Wortman,[1]* P. Rogers,[1] Y. Charoenvit,[1] A. McDermott,[2] M. Leef,[1] M. Sedegah[1] and R. L. Beaudoin[1]†

[1]*Infectious Diseases Program Center, Naval Medical Research Institute, Bethesda, Maryland 20814-5055, U.S.A., and* [2]*Biomedical Research Institute, Rockville, Maryland, 20852, U.S.A.*

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Genomic DNA isolated from *Plasmodium yoelii* (strain # 17XNL) was prepared by partial digestion and cloned in *Escherichia coli* TB-1 with pUC18 plasmid. Antigen-producing recombinants were detected by a battery of monoclonal antibodies against antigens of the sporozoite stages. Four clones producing stage-specific sporozoite antigens were identified. One produced *P. yoelii* circumsporozoite protein, and three produced other *P. yoelii* sporozoite antigens.

Key words: sporozoite antigens; *Plasmodium yoelii*; recombinant DNA; monoclonal antibodies; rodent malaria.

Introduction

Despite the rapid advances in the development of a recombinant vaccine against *Plasmodium falciparum* sporozoites, there is still an urgent need for a reliable rodent model to carry out complex vaccine protocols difficult to perform in primates or man. The purpose of this research was to clone the *P. yoelii* genes coding for sporozoite antigens that have potential as vaccine candidates in a rodent model.

Results

Four positive clones (B10, B85, B143 and B155) were identified from a genomic library containing 10^5 clones. Each clone reacted with one of three Mabs (NYS5, NYS4 or NYS1). These Mabs recognized different epitopes on sporozoite antigens, produced different IFA staining patterns, and detected antigens of different molecular weights. Clone B155 reacted with NYS1, the protective Mab recognizing circumsporozoite (CS) antigen; clones B85 and B143 reacted with NYS4, while B10 reacted with NYS5.

Restriction digests of clones B155, B10, B85 and B143 were each analysed by

† Present address: Molecular Biosystems Incorporated, 11180-A Roselle St., San Diego, CA 92121, U.S.A.
* Author to whom correspondence should be addressed.

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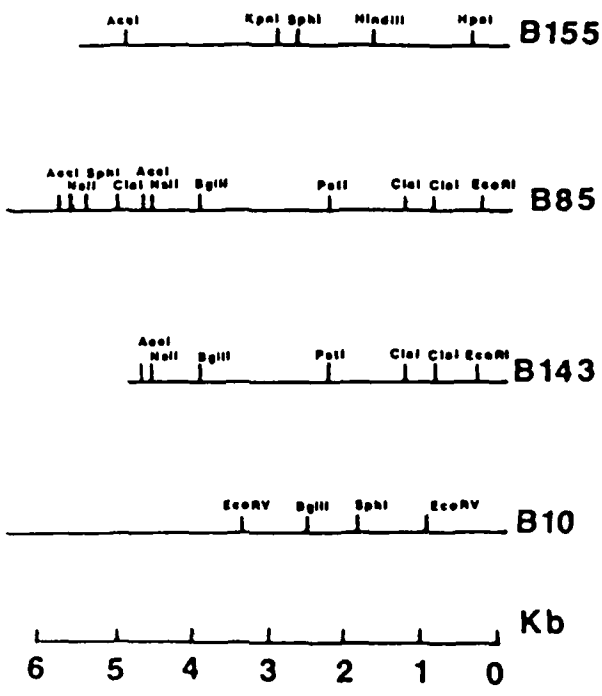

Fig. 1. Restriction maps of four cloned DNA segments expressing three different sporozoite specific antigens of *Plasmodium yoelii*.

agarose gel electrophoresis. The restriction patterns of B155, B10 and B143 were different, indicating that each of their DNA sequences is unique whereas that of B143 was identical with the pattern obtained with the corresponding portion of B85 showing these last two clones contain identical parts of the same gene (Fig. 1).

The purified insert DNA from B10 (Fig. 2) and B155 (Fig. 3) hybridized only with

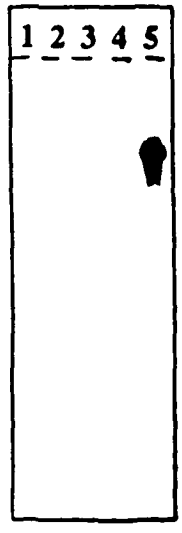

Fig. 2. Southern blot of three inserts of *Plasmodium yoelii* DNA cloned into pUC18 hybridized with ^{32}P nick-translated purified insert of clone B10. Lane 1 empty, linear pUC18 (lane 2), clone B155 cut with *Eco*RI and *Pst*I (lane 3) clone B143 cut with *Kpn*I and *Hin*dIII (lane 4), and clone B10 cut with *Bam*HI (lane 5).

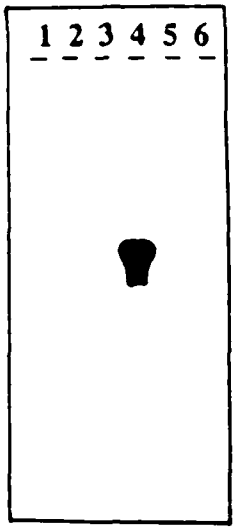

Fig. 3. Southern blot of three inserts of *Plasmodium yoelii* DNA cloned into pUC18 hybridized with ^{32}P nick-translated purified insert of clone B155. Lanes 1 and 2 empty, linear pUC18 (lane 3), clone B155 cut with *Eco*RI and *Pst*I (lane 4), clone B143 cut with *Kpn*I and *Hin*dIII (lane 5), clone B10 cut with *Bam*H (lane 6).

DNA from the same clone. Neither cross-hybridized with the DNA from either of the other two clones or from pUC18. Recombinant plasmids of the positive clones were extracted, retransformed and re-screened to confirm that the DNA responsible for production of the detected antigen was plasmid-associated.

The expressed recombinant peptide produced by clone B155 inhibited the reactivity of NYS1 in an IFA inhibition assay using fresh sporozoites as the antigen. The results of this test also indicate that Mabs NYS4 and NYS5 recognize antigens other than the CS protein, since they were not inhibited by the CS recombinant peptide (Table 1).

Discussion and conclusions

The conclusion that three of the four cloned *P. yoelii* DNA fragments identified by the Mabs code for three different sporozoite antigens is supported by the following evidence: (1) the clones were identified by three different characterized Mabs; (2) the

Table 1 Inhibition of monoclonal antibody immunofluorescence reactivity with *Plasmodium yoelii* sporozoites by the product of the recombinant *P. yoelii* circumsporozoite gene

Monoclonal antibody[a]	Recombinant clone detected	IFA antigen (sporozoite)	Inhibition
NYS1	B155	*P. yoelii*	+
NYS4	B85, B143	*P. yoelii*	–
NYS5	B10	*P. yoelii*	–
NBS1	Negative control	*P. berghei*	–
NFS2	Negative control	*P. falciparum*	–

[a] NBS1 is a Mab directed against the CS antigen of *P. berghei* while NFS2 is a Mab directed against the CS antigen of *P. falciparum*. The IFA specificities and other characteristics of Mabs NYS1, NYS4 and NYS5 have been reported previously.[3]

restriction maps of the DNA from the three clones were different; (3) there was no cross-hybridization of the insert DNA from any of the three clones; (4) the expressed recombinant peptide from clone B155 inhibited only Mab NYS1 in an IFA inhibition test.

Clone B155 is known to express the CS antigen recognized by a protective, CSP positive Mab, NYS1. The entire gene coding for this antigen is contained in clone B155 and this gene has now been sequenced and subcloned into high expression vectors.[1]

The product of clone B155 provides a recombinant vaccine rodent model, based on the CS antigen. The other clones express two additional stage-specific sporozoite antigens never before reported for any species of *Plasmodium*.

Materials and methods

Purification of parasite DNA. Parasitemias in mice infected with *P. yoelii* (strain 17XNL) were allowed to reach 25% infected red blood cells before blood was collected. 20 ml of heparinized blood from 30 infected mice were mixed with 0.85% saline and passed over a CF-12 cellulose column to remove white blood cells. The blood was sedimented (2500 *g*), the plasma removed, and the red blood cells lysed in 0.1% saponin in 1 × SSC (0.15 M sodium chloride, 0.015 M sodium citrate). Freed parasites were washed in 1 × SSC, resuspended, and lysed in a solution containing 1 × SSC, 1% sarkosyl and proteinase K (1 mg/ml), then incubated at 37°C for 1 h. CsCl (1.1 g/ml) was added to the lysate followed by 600 μg/ml ethidium bromide (ETBR). Following ultracentrifugation (82 000 *g*, 44 h) the DNA was collected, extracted free of ETBR, and quantitated by spectrophotometry.

Preparation of 9 Kb fragments of eukaryotic DNA. A series of *Sau*3A digestions were made, which ranged from complete to minimal. The pooled digests were sedimented through a 10–40% sucrose gradient for 20 h at 62 500 *g* at 20°C.[2] Fractions (750 μl) were collected and assayed, and those containing DNA fragments greater than 5 kb were pooled, dialysed, and ethanol precipitated.

Preparation of vector DNA. pUC18[3] was digested with *Bam*HI; the completeness of the digest was assessed by agarose gel electrophoresis and cell transformation. Linear pUC18 was treated with bacterial alkaline phosphatase which was subsequently inactivated by proteinase K digestion and phenol extraction.

Construction of genomic library. The size fractionated *P. yoelii* DNA was then ligated into pUC18 in 1 : 1, 1 : 2 and 1 : 3 ratios (vector : insert) at a final DNA concentration of 50 μg/ml. The ligated DNA was diluted 1 : 5 in Tris–EDTA (TE) buffer prior to transformation into *E. coli* TB-1 cells rendered competent by the $CaCl_2$ procedure of Cohen, Chang and Hsu.[4] Following heat shock and a subsequent 1 h incubation at 37°C, 50 μl aliquots were plated onto Luria-Bertani agar (LB) ampicillin (50 μg/ml) plates[5] containing isopropylthiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). White colonies were transferred to LB-ampicillin master plates. Forty random clones were grown overnight in LB broth, and lysed by the method of Holmes and Quigley.[6] Mean insert length was 9 kb.

Identification of recombinant clones. A total of 12 000 clones were prepared for monoclonal antibody screening. Each clone was inoculated into a microtiter well containing 225 μl of LB ampicillin broth and grown overnight at 37°C. Groups of three clones (70 μl each) were pooled into wells of microtiter plates, lysed by the addition of sodium dodecyl sulfate (1% final concentration) and agitated for 30 min at 37°C. Following lysis, RNAse A and DNAse I were added to final concentrations of 50 μg/ml and 30 μg/ml respectively, and incubated with agitation for 30 min at 37°C. Lysate aliquots (100 μl) were added to wells of a Hybridot manifold (BRL) containing a nitrocellulose sheet, and allowed to stand 30 min before being drawn through the nitrocellulose. The sheets were dried at room temperature and screened with monoclonal antibodies. Five stage-specific *P. yoelii* antisporozoite Mabs (NYS1 through NYS5) were used for screening the recombinant clones.[7] All five of these Mabs produced a positive reaction with the sporozoite stage by immuno-fluorescence but were negative with the other

stages tested. NYS1 and NYS2 were IgG3 protective and positive in a circumsporozoite precipitation (CSP) test which determines surface reactivity. NYS3 was a non-protective IgM antibody but was CSP positive. NYS4 was an IgM non-protective antibody, which was CSP negative. NYS5 was an IgG1, non-protective antibody and was CSP negative.

The nitrocellulose sheets containing lysates from recombinant clones were blocked in 3% BSA in phosphate buffered saline (PBS), pH 7.2, for 45 min with continuous agitation on a rocking platform and washed three times in PBS containing 0.05% tween 20 (PBS-tween). The sheets were then incubated for 2 h at room temperature with the pool of 5 Mabs (NYS1 through NYS5) diluted to 1:100 in PBS containing 20% fetal bovine serum (PBS-FCS). The sheets were washed three times in PBS-tween and incubated for 2 h with affinity-purified rabbit anti-mouse immunoglobulin at a final concentration of 2 μg/ml in PBS-FCS. The sheets were washed three times in PBS-tween, and incubated for 2 h in ^{125}I staphylococcal protein A (New England Nuclear, specific activity 8.8 μCi/μg purified Ig) at a concentration of 2×10^5 cpm/ml in PBS-FCS. The sheets were washed extensively with PBS-tween, air-dried and exposed to Kodak X-OMAT AR (Eastman Kodak) for 3 days at −70°C before the films were developed. Individual colonies from the pools giving a positive signal were screened separately with the pooled Mabs. Positive clones were then screened with each of the five Mabs.

Restriction digests and Southern blots of positive clones. To determine whether the clones represented unique gene segments, restriction digests of clones B10, B85, B143 and B155 were each analysed by agarose gel electrophoresis and blotted onto nylon.[5] Southern blots of B10, B143 and B155 were hybridized with ^{32}P nick-translated DNA from clones B10 and B155. Hybridization was performed at 50°C overnight, then preparations were washed in 0.1 × SSC and 0.1% (w/v) SDS for 2 h at 55°C with four solution changes. Hybridized blots were exposed overnight to Kodak X-OMAT XAA-5 at −70°C before the films were developed.

Immunofluorescence inhibition test. The expressed recombinant peptides produced by this CS gene have been used in an immuno-fluorescence inhibition test. Serial dilutions of the antibodies were mixed with equal quantities of recombinant peptide, incubated at room temperature for 1 h and then at 4°C overnight. The supernatants were transferred to wells containing dried *P. yoelii* sporozoites, incubated for 30 min, washed with PBS and then incubated with fluorescein conjugated rabbit anti-mouse IgG (Miles). After further washing, they were examined using an American Optical UV microscope.

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References

1. Theisin TW, Gross M, Young J, Charoenvit Y, Rogers PL, McDermott A, Wortman A, Sedegah M, Leef M, Beaudoin RL. Cloning and expression of the *Plasmodium yoelii* circumsporozoite gene. Unpublished
2. Wortman AT, Somerville CC, Colwell RR. Chitinase determinants of *Vibrio vulnificus*: gene cloning and applications of a chitinase probe. Appl Environ Microbiol 1986; 52: 142–5.
3. Yanisch-Perron C, Viera J, Messing J. Improved M13 phage cloning vectors and host strains: nucleotide sequences of M13 mp 18 and pUC19 vectors. Gene 1985; 33: 103–19.
4. Cohen SN, Chang ACY, Hsu L. Nonchromosomal antibiotic resistance in bacteria: Transformation of *E coli* by R-factor DNA. Proc Natl Acad Sci USA 1979; 69: 2110–4.
5. Maniatis T, Fritsch EF, Sambrook J. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY Cold Spring Harbor Laboratory Press, 1982.
6. Holmes DS, Quigley M. A rapid boiling method for the preparation of bacterial plasmids. Analyt Biochem 1981; 114: 193.
7. Charoenvit Y, Leef MF, Yuan LF, Sedegah M, Beaudoin RL. Characterization of *Plasmodium yoelii* monoclonal antibodies directed against stage-specificsporozoite antigens. Infect Immun 1987; 55 604-8